AMYLASE PRODUCTION BY A THERMOPHILIC BACTERIUM, *Bacillus licheniformis* BT5.9 ISOLATED FROM CHANGAR HOT SPRING, MALANG, INDONESIA

By

HAN LI ZHU

Thesis submitted in fulfillment of the requirement for the degree of Master of Science

April 2012

ACKNOWLEDGEMENTS

My heartiest and deep felt gratitude is for my supervisor, Professor Darah Ibrahim, who has paid her heart and soul guidance on my project. I make a grateful acknowledgement for her technical and encouragement. Also my deeply thanks to Professor Ahmad Shukri Mustapa Kamal who helped me to get Master of Science Degree offer letter.

My thanks to my labmates, Mazwin, Nur Aqilah, Suraya, Lee Kok Chang, Lim Sheh Hong, Hassan, Najiha, Syarifah, Syazni, Aishah, Sumathi, Chee Mei Sing, Choo Mang Ling, Anu and all of the person who helped me throughout my study, my project and this thesis. My thanks also to the School of Biological Sciences for supporting me with the materials and the equipments needed for my project. My thankfulness extended to the International Office of USM and Malaysia Immigration Office as they had helped me to get and change Student Visa.

Finally, I would like to pay special respect and thanks to; the Manager of the Student International House, all the members of the library, all my friends' family members whose open house I had been invited, all the staffs of USM and Malaysia government. Thanks again to everyone who has given me helps during my study in Malaysia.

Table of contents

List of Tables

List of Figures

Penghasilan amilase oleh bakteria termofili, *Bacillus licheniformis* BT5.9 yang dipencil dari kolam Air Panas Changar, Malang, Indonesia

Abstrak

Kajian ini telah dijalankan untuk mencari mikroorganisma termofili yang mempunyai sifat khusus sebagai sumber enzim amilase termostabel. Enam sampel yang terdiri daripada tanah, air dan slaj yang dikutip dari kolam air panas Changar di Malang, Indonesia telah dikaji. Sampel tersebut diambil dari kolam air panas yang terletak di kawasan gunung berapi yang mempunyai suhu antara 49.5 hingga 56.0 ℃ dan pH antara 5.7 hingga 6.5. Tiga belas daripada 34 pencilan bakteria termofili yang dipencilkan menunjukkan potensi sebagai penghasil amilase dan pencilan BT5.9 yang dikenalpasti sebagai *Bacillus licheniformis* BT5.9 menerusi ujian biokimia dan molekul 16S rRNA telah dipilih sebagai penghasil amilase terbaik. Penambahbaikan keadaan pengkulturan (pH awal medium 5.0, suhu pengkulturan 50 ℃, kelajuan goncangan 100 psm dan saiz inokulum sebanyak $1.7x10^8$ sel/ml) dan komposisi medium (g/L: 20.0 g kanji terlarut, 2.5 g ekstrak yis, 1.0 g $MgSO_4 \cdot 7H_2O$, 3.0 g Na_2HPO_4, 0.1 g NaCl, 0.3 g $FeSO_4$) telah menghasilkan sebanyak 1.06 U/ml amilase pada masa pengkulturan 18 jam. Terdapat peningkatan sebanyak 222.94% penghasilan amilase selepas penambahbaikan keadaan pengkulturan dan komposidi medium. Kajian ini juga menunjukkan yang penghasilan enzim tidak bergantung kepada pertumbuhan sel. Penulenan pula telah menghasilkan satu jalur tunggal pada SDS-PAGE menunjukkan amilase tulen telah

diperolehi dengan berat molekul sekitar 24.55 kDa. Aktiviti amilase tulen didapati optimum pada suhu 70℃ dan pH 6.0 dengan 100% aktiviti selama 60 minit pada suhu dan pH tersebut.

Amylase production by a thermophilic bacterium, *Bacillus licheniformis* BT5.9 isolated from Changar Hot Spring, Malang, Indonesia.

ABSTRACT

This study was carried out in order to search for thermophilic microorganisms with special interest as a source of thermostable amylases. Six samples which consisted of soils, water and sludge were taken from Changar Hot Spring in a volcanic area of Malang, Indonesia. The samples had temperature of 49.5 to 56.0^{o}C and pH of 5.7 to 6.5. Thirteen out of 34 of bacterial isolates were obtained from those samples and exhibited potential amylase producer, and isolate BT5.9 which was identified as *Bacillus licheniformis* BT5.9 through biochemical tests and 16S rRNA molecular analysis was chosen as the best amylase producer. The improvement of cultural conditions (initial medium pH of 5.0, cultivation temperature of 50^{o}C, agitation speed of 100 rpm and inoculum size of $1.7x10^{8}$ cells/ml) and medium compositions (g/L: 20 g soluble starch, 2.5 g yeast extract, 1.0 g $MgSO_4 \cdot 7H_2O$, 3.0 g Na_2HPO_4, 0.1 g NaCl, 0.3 g $FeSO_4$) produced the highest amylase production about 1.06 U/ml at 18 hours of cultivation time. There was an increment of 222.94% of amylase production obtained after the improvement of cultivation conditions and medium compositions. The study revealed that the enzyme production was not growth dependant. Purification process produced a single band on SDS-PAGE indicated that the purified amylase was obtained with a molecular weight of about 24.55 kDa. The activity of purified amylase was found optimum at the temperature of 70^{o}C and pH of 6.0 with 100% activity at those parameters for 60 minutes.

Chapter 1: Introduction

1.1 Thermostable enzymes

Thermostable enzymes are more versatile than thermolabile as they have higher operational stability and longer self-life at elevated temperatures (Niehaus, *et al.,* 1999). Besides, thermostable enzymes also have been reported to have higher stability to pH (acidic or alkaline), detergents and organic solvents (Vieille *et al.,* 1996). Therefore, the thermophilic microorganisms are of special interest for producing thermostable enzymes. Due to the increasing demand for thermostable enzymes in various industries, therefore it has to be isolated, produced and characterized from different sources.

Thermostable enzymes from microorganisms have found a number of commercial applications because of their overall inherent stability (Demirijan *et al*., 2001). The most widely used thermostable enzymes in industry are the amylases especially in the starch industy (Demirkan *et al*., 2005). They are among the most important enzymes and are of great significance in present-day biotechnology. Although they can be derived from several sources, such as plants, animal and microorganisms; amylases from microbial sources generally meet industrial demands.

From the definition of thermostable amylases, the enzymes usually have two main characteristics, that are thermostable and enzyme characteristics. The other

important characteristics included high efficiency, stable in normal environment, storage and transportation conditions are easy to control, low cost of industrial production, and pure enough or there is no other deleterious byproducts in enzyme preparations. Those are the characteristics in thermostable amylases that the industries are looking for. Even though, it seems to be very easy to get, but there are various difficulties and problems to achieve them. The production of thermostable amylases is a complicated work.

1.2 Amylases

Amylases are enzymes which hydrolyze starch molecules to give diverse products including dextrins and progressively smaller polymers composed of glucose units. These enzymes have a great significance with biotechnological applications in bread and baking, food, textile, and paper industries (Pandey *et al.,* 2000). The starch industry is one of the largest users of enzymes for the hydrolysis and modification of this useful raw material. The starch polymer, like other such polymers, requires a combination of enzymes for its complete hydrolysis. These include α-amylases, glucoamylases or β-amylases and isoamylases or pullulanases. The enzymes are classified into endo-acting and exo-acting enzymes. α-amylase is an endo-acting enzyme and hydrolyses linkages in a random fashion and leads to the formation of linear and branched oligosaccharides, while the rest are exo-acting enzymes and attack the substrate from the non-reducing end, producing oligosaccharides and monosaccharides. The starch hydrolytic enzymes

comprise 30% of the world's enzyme consumption (van der Maarel *et al*., 2002). Developments in the starch-processing industry require continued discovery and development of new enzymes. Until now all commercial enzymes have been derived from cultivated bacteria or fungi. Notably, intensive research has been performed aiming at the isolation of unique and novel thermostable and thermoactive amylases from thermophilic and hyperthermophilic microorganisms, therefore allowing more industrial processes to run at higher temperatures (Niehaus *et al.,* 1999). Thermostable α-amylases have extensive commercial demand and applications in industry with extensive biotechnological applications in food, in starch processing, brewing and sugar production (Leveque *et al.,* 2000; Haq *et al*., 2010), desizing in textile industries, paper industries and in detergent manufacturing processes (Hewitt and Solomons, 1996). Thermostability is a desired characteristic of most of the industrial enzymes.

1.3 Objectives of research

In order to search for new thermophilic bacteria that can produce thermostable amylase, a study was carried out by using samples of soils collected from Changar Hot Spring, Surabaya, Indonesia. The objectives of research are;

i. to isolate and identify thermophilic bacteria isolated from the soil samples collected from Changar Hot Spring, Malang, Indonesia that can produce amylase.

ii. to improve the cultural conditions and medium compositions for maximal

amylase production by the selected bacterial culture (*Bacllus licheniformis* BT5.9).

iii. to purify and characterize the amylase produced by the selected bacterial culture (*Bacllus licheniformis* BT5.9).

Chapter 2: Literature Review

2.1 Thermophiles

Thermophiles are organisms which thrives at relatively high temperatures, above 50 ℃. Thermophiles are found in holes of geothermally heated regions of the earth such as hot springs like those in Yellowstone National Park, from marine and terrestrial geothermally-heated habitats including shallow terrestrial hot springs, hydrothermal vents, sediments and soils from volcanic areas and deep sea hydrothermal vents as well as decaying plant matter such as peat bogs and compost (Bertoldo and Antranikian, 2002). As a prerequisite for their survival, thermophiles contain enzymes that can function at high temperature. Some of these enzymes are used in molecular biology such of the heat-stable DNA polymerases for polymerase chain reaction and also in washing agents. Thermophiles are classified into obligate and facultative thermophiles. Obligate thermophiles (extreme thermophiles) require such high temperatures for growth, while facultative thermophiles (moderate thermophiles) can thrive at high temperatures but also at lower temperatures (below 50 ℃). Hyperthermophiles are particularly extreme thermophiles for which the optimal temperatures are above 80 ℃.

Thermophiles and hyperthermophiles have been isolated from many natural habitats, including continental mud-pools (Solfatara), hot springs and geysers (Yellowstone National Park), and deep sea sediment or vents, such as black

smokers (Mid-Atlantic Ridge). In addition, they have been isolated in heated industrial environments, like the outflows of geothermal power plants and sewage sludge systems (Kristjansson and Hreggvidsson, 1995; Stetter, 1999; Bertoldo and Antranikian, 2002). Most of the thermophiles and hyperthermophiles are chemolithoautotrophs and fix CO_2 by chemosynthesis. Besides H_2 as an important electron donor, sulfide, sulfur and ferrous iron can also be used for donoring the elcctron. The majority of thermophiles and hyperthermophiles grow anaerobically, although some of them are facultative, or even strictly aerobic, yet at reduced oxygen concentrations. Several thermophiles and hyperthermophiles are facultative heterotrophs, being able to switch from autotrophic to heterotrophic growth when organic material is presented. Most of them are well equipped to degrade peptides, and many are even capable of growing on a variety of simple and complex carbohydrates (Sivaramakrishnan *et al*., 2006; de Souza and Magalhaes, 2010).

Many thermophiles and hyperthermophiles grow well on both α- and β-linked carbohydrates (Sivaramakrishnan *et al.,* 2006). In term of metabolisms, carbohydrates are preferably used as di- or polysaccharides, because of the thermal liability of monosaccharides, as was observed in the fermentation of glucose versus cellobiose by *Pyrococcus furiosus* (Blamey and Adam, 1993; Veieille and Zeikus, 2001).

2.2 Thermophilic microorganisms

Thermophilic microorganisms prefer temperatures above 55^{o}C and can tolerate temperatures up to 75-80^{o}C. Extreme thermophiles even can live in boiling water.

Thermophiles are found in various geothermally heated regions of the Earth such as hot springs and deep sea hydrothermal vents, as well as decaying plant matter such as peat bogs and compost.

2.2.1 Thermophilic bacteria

Bacteria are especially abundant and are usually divided into several classes based upon the temperatures at which they grow best. The low temperature bacteria are the psychrophiles, which can grow at temperatures down to -10^{o}C but whose optimum temperature is 15^{o}C or lower. The mesophiles live at medium temperatures, 20-45^{o}C, and include human pathogens. Thermophiles thrive above 50^{o}C, and some live at or even above the boiling point of water.

Strains of thermophilic bacteria have been identified with optimum temperatures ranging from 50^{o}C to an incredible 105^{o}C, and many temperatures in between. Thermophilic bacteria occur naturally in hot springs, tropical soils, compost heaps, excrement, hot water heaters (both domestic and industrial). Thermophilic bacteria were first isolated in 1879 by Miquel, who found bacteria capable of growing at 72^{o}C (Phillips and Perry, 1976). He found these bacteria in soil, dust, excrement, sewage, and river mud. Since the findings, thermophilic bacteria have

been isolated from soil, and those bacteria that readily thrived at high temperatures, but not at room temperature. Since then many thermophilic bacteria have been isolated and characterized, and among bacteria, *Bacillus* sp. is the most isolated species, such as *B. subtilis, B. stearothermophilus, B. licheniformis, B. amyloliquefaciens* (Haki and Rakshit, 2003). Table 2.1 shows some of the species of *Bacillus* that have been reported to produce amylases.

Table 2.1: Some of the *Bacillus* spp. that have been reported to produce amylases

Species	Optimal temperature (℃)	Optimal pH	References
Bacillus sp. WA21	85	5-9	Asad *et al*., 2011
Bacillus amyloliquefaciens	70	7.0	Underkofler (1976)
Bacillus licheniformis	100	6.0-6.5	Viara *et al*., (1993)
Bacillus stearothermophilus	70-80	5.0-6.0	Vihinen *et al.,* (1990)
Bacillus stearothermophilus	70	-	Jeayoung *et al.*, (1989)
Bacillus subtilis	70	7.0	Canganella *et al*., (1994)
Bacillus megaterium	60	7.0	Oyeleke *et al*., (2010)
Bacillus sp. 1-3	60	7.0	Goyal *et al*., (2005)
Bacillus sp. ANT-6	60	10.5	Burhan *et al*., (2003)
Bacillus megaterium	60	7.0	Oyeleke & Oduwole, (2009)
Bacillus macerans	60	7.0	Oyeleke & Oduwole, (2009)
Bacillus coagulans	60	7.0	Oyeleke & Oduwole, (2009)
Bacillus pumilus	60	7.0	Oyeleke & Oduwole, (2009)
Bacillus Thermoleovorans ID-1	70	7.0	Mamo *et al*., (1999)
Bacillus circulans	60	-	Poonam & Dalel (1995)
Bacillus cereus var *mycoides*	50	-	Takasaki (1976)
Bacillus sp. KYJ963	50	7.5	Young *et al*., (2001)
Bacillus licheniformis	90	7.0	Vaseekaran *et al*.,(2010)

2.2.2 Thermophilic yeasts

Temperature is one of the most important parameters affecting the growth and survival of microorganisms. Most microorganisms are mesophiles and occupy temperature niches that are not regarded as extreme. Yeasts are ascomycetous or basidiomycetous fungi that reproduce vegetatively by budding or fission, and that form sexual states which are not enclosed in a fruiting body (Boekhout and Kurtzman, 1996). The yeast species are all characterized by a similar set of features, both morphological and physiological. It has been proposed that a thermophilic yeast should be defined as a yeast which has a minimum temperature for growth of 20^{o}C and no restriction on the maximum temperature for growth. If this definition is used, all of the species in this category are enteric yeasts isolated from digestive tracts of various animals. These yeasts, which have growth temperature limits between 20 and 46^{o}C (Travassos and Cury, 1971), and they include the respiratory-deficient organisms *Candida slooffii, Torulopsis pintolopesii* and the respiratory-competent organisms *Saccharomyces telluris* and *Torulopsis bovina* (Deegenaars and Watson, 1998). Heat shock response in the thermophilic enteric yeast). However, there are a few thermophilic yeasts that can utilize carbohydrate such as *Kluveromyces marxianus* (Barron *et al*., 1994), *Arxiozyma telluris* (Watson *et al.*, 1978) and *Candida thermophila* (Shin *et al.*, 2001).

2.2.3 Thermophilic fungi

Thermophilic fungi are a small assemblage that have a minimum temperature of growth at or above 20℃ and a maximum temperature of growth extending up to 60 to 62°C (Maheswari *et al*., 2000). As the only representatives of eukaryotic organisms that can grow at temperatures above 45°C, thermophilic fungi are valuable experimental systems for investigations of mechanisms that allow growth at moderately high temperature yet limit their growth beyond 60 to 62°C. Although widespread in terrestrial habitats, they have remained under explored compared to thermophilic species of eubacteria and archaea. However, thermophilic fungi are potential sources of enzymes with scientific and commercial interests (Silva *et al*., 2005). Thermophilic fungi can be grown in minimal media with metabolic rates and growth yields comparable to those of mesophilic fungi. Studies of their growth kinetics, respiration, mixed-substrate utilization, nutrient uptake, and protein breakdown rate have provided some basic information not only on thermophilic fungi but also on filamentous fungi in general (Maheswari *et al*., 2000). Some species have the ability to grow at ambient temperatures if cultures are initiated with germinated spores or mycelial inoculum or if a nutritionally rich medium is used (Maheswari *et al*., 1987).

Thermophilic fungi have a powerful ability to degrade polysaccharide constituents of biomass. The properties of their enzymes show differences not only among species but also among strains of the same species. Their extracellular enzymes

display temperature optima for activity that are close to or above the optimum temperature for the growth of organism and, in general, are more heat stable than those of the mesophilic fungi. Some extracellular enzymes from thermophilic fungi are being produced commercially, and a few others have commercial prospects. Genes of thermophilic fungi producing lipase, protease, xylanase, and cellulase have been cloned and overexpressed in heterologous fungi, and pure crystalline proteins have been obtained for elucidation of the mechanisms of their intrinsic thermostability and catalysis (Boel *et al*., 1986; Derewenda *et al*., 1992). By contrast, the thermal stability of the few intracellular enzymes that have been purified is comparable to or, in some cases, lower than that of enzymes from the mesophilic fungi. Although rigorous data are lacking, it appears that eukaryotic thermophily involves several mechanisms of stabilization of enzymes or optimization of their activity, with different mechanisms operating for different enzymes. Among the thermophilic fungi that heve been reported to produce enzymes especially amylases are *Thermomyces lanuginosus* (Jensen *et a*l., 2002; Kunamneni *et al*., 2005a), *Myceliophthora thermophilia* D14 (ATCC 48104) (Sadhukan *et al*., 1990) and *Rhizomucor pusillus* A13.36 (Silva *et al*., 2005).

2.2.4 Thermophilic actinomycetes

Actinomycetes comprise a large and diverse group of largely mycelial bacteria, many of which are important ecologically and are exploited commercially for the production of natural products such as antibiotics and enzymes. Thermophilic

actinomycetes are also known but these are relatively poorly studied compared to the predominant mesophilic genera (Edwards, 1993). Actinomycetes are Gram positive bacterial-like microrganisms that tend to form filaments and may also produce airborne spores. They have been called "actinomycetes" because of a superficial resemblance to fungi. However, the actinomycetes are prokaryotic (have no organized nucleus) and are physiologically and chemically related to bacteria. They are also much smaller than fungi (Edwards, 1993) and can be found abundantly in soils, silos, corn mills, closed stables, bagasses, house dust and home as well as in industrial air conditioning systems.

Actinomycetes can be aerobic or anaerobic, although the thermophilic forms are primarily aerobic. The majority of actinomycetes are mesophilic, growing at temperatures ranging from 18^{o}C to 45^{o}C. A few of them are thermophilic (Kusrer and Locci, 1963; Kuo and Hartman, 1966), which means that they have an optimal range for growth between 40^{o}C and 80^{o}C (Tortora, 2007). Common species of thermophilic actinomycetes include the genera of *Streptomyces*, *Thermomonospora* and *Thermoactinomyces* such as *Thermoactinomyces vulgaris* (Allam *et al.*, 1975), *Thermomonospora curvata* (Collins *et al*., 1993; Glymph and Stutzenberger, 1977), *Streptomyces clavifer* (Hoque *et al*., 2006), *Saccharopolyspora rectivirgula* and *Streptomyces albidoflavus* (Narayana and Vijayalakshmi, 2008) have been reported to produce enzymes namely amylases, pullulanase and glucosyltransferase activities.

2.3 Importance of enzymes from thermophiles

Demand of thermostable enzymes has increased tremendously due to its high thermostability and feasibility to the processes involved. One of the obvious advantages of carrying biotechnological processes at elevated temperatures is reducing the risk of contamination by common mesophiles. Besides, an operation at the high temperatures has significant influence on the bioavailability and solubility of organic compounds and thereby provides efficient bioremediation (Becker, 1997). Other values of elevated process temperatures include higher reaction rates due to decreased viscosity and hence increased diffusion coefficient of substrates leading to the favorable equilibrium displacement in endothermic reactions (Kumar and Swati, 2001). Such enzymes can also be used as models for the understanding of thermostability and thermoactivity tha is useful for protein engineering (Haki and Rakshit, 2003).

The enzymes isolated from some thermophiles have proven to be of great use in the biotechnology industry, able to function under conditions that would denature enzymes taken from most normal organisms. The most commonly used DNA polymerase for the polymerase chain reaction technique is Taq DNA polymerase, originally isolated from *Thermus aquaticus*, a bacterial species found in surface aquatic locations such as Yellowstone National Park hot springs. For a few polymerase chain reaction (PCR) applications, the lack of proofreading by Taq DNA polymerase is a problem. The DNA polymerase from *Thermococcus*

litoralis is shown to have a proofreading exonuclease activity (Mattila *et al*., 1991). Another heat stable polymerase comes from the organism *Pyrococcus furiosus* (Pfu). This organism grows optimally at 100℃, making it a hyperthermophile. Taq DNA polymerase is adequate for most PCR, but Hamilton *et al.,* (2001) reported that higher fidelity thermostable DNA polymerases such as Vent account for as much as 30% of DNA polymerase sales.

2.4 Ability of thermophilic microorganisms to survive and produce enzymes at high temperatures

Microorganisms have the ability to adapt to the conditions in which they have to live and survive. In thermophilic microorganisms, they have thermostable proteins which resist denaturation and proteolysis (Kumar and Nussinov, 2001). Specialized proteins known as chaperonin are produced by these microorganisms which assist, after their denaturation to refold the proteins to their native form and restore their functions. Their cell membranes contain saturated fatty acids which provide a hydrophobic environment for the cell and maintain the cell rigidity at elevated temperatures (Herbert and Sharp, 1992). Furthermore, the thermophilic and hyperthermophilic microorganisms have lipids linked with ether on the cell wall, which is responsible for heat resistance. This layer is much more heat resistant than a membrane formed of fatty acids (De Rosa *et al*., 1994). Recently, tetraether membrane lipids were reported in a thermoacidophilic euryarchaeota *Candidatus* "*Aciduliprofundum boonei*" (Schouten *et al*., 2008). In addition to the structural adaptations of cell wall and cell membrane, the DNA of thermophilic

microorganisms contains reverse DNA gyrase, which enhance the melting point by producing positive super coils in the DNA (Lopez, 1999). In *Sulfolobus solfataricus* a small DNA binding protein, Sso7d, not only imparts thermostability to the DNA but also promotes the annealing of complementary strands above the melting point and the ATPase-dependent rescue of the aggregated proteins (Mai *et al*., 1998). Thermophiles are reported to have proteins which are thermostable and resist denaturation and proteolysis. In addition, thermophilic bacteria, actinomycetes and archae adapt to high temperatures by increased electrostatic, disulphide and hydrophobic interactions in their proteins (Kumar and Nussinov, 2001). In addition to the above strategies, certain specialized proteins, known as 'chaperons', are produced by these organisms, which help to refold the proteins to their native form and restore their functions . Stability and folding pattern of the ferredoxin from a hyperthermophilic archaeon *Acidianus ambivalens* has been studied using Guanidine HCl as a chemical denaturant (Wittung-Stafshede *et al*., 2000). Certain thermophilic enzymes are stabilized by certain conformational changes. However, presence of certain metals, inorganic salts and substrate molecules are also reported to impart the thermostability (Leppanen *et al*., 1999). Based on the thermal behavior of these enzymes, the equilibrium model has been described to reveal the effect of temperature on enzyme activity by reversible active-inactive transition states. Due to the increasing demand of highly thermostable enzymes, certain computational algorithms and bioinformatic tools are designed to predict protein rigidity and stability in order to improve the

thermostability. Protein stabilization can be carried out by site-directed mutagenesis (Parthiban *et al.*, 2006). The other powerful protein engineering method to add to the thermostability is directed evolution which involves gene shuffling through which sequence optimization leads to a combination of novel traits (Neylon, 2004).

2.5 Amylase

Amylases are enzymes that catalyzes the hydrolysis of starch molecules to give various products including dextrins and other smaller polymers such as maltose and maltotriose which composed of monomer of glucose unit (Sivaramakrishnan *et al*., 2006). Amylases can be grouped as α-amylase, β-amylase and γ-amylase.

The α-amylases (EC 3.2.1.1; 1,4-α-D-glucan glucanohydrolase) family comprises a group of enzymes with variety of different specificities that all act on one type of substrate that is glucose residues linked through an α-1,1, α-1,4, α-1,6 glycosidic bonds. Members of this family share a number of common caharacteristic properties (van de Maarel *et al*., 2002). Amylases can be grouped into two groups, endo-amylases and exo-amylases. Endo-amylases catalyze hydrolysis in a random manner in the interior of the starch molecule producing linear and branched oligosaccharides of various chain lengths. Exo-amylases on the other hand act from the non-reducing end successively resulting in short end products (Gupta *et al.*, 2003).

α-amylases are starch-degrading enzymes that catalyze the hydrolysis of internal α-1,4-o-glycosidic bonds in polysaccharides with the retention of α-anomeric configuration in the products. Most of the α-amylases are metalloenzymes which require calcium ion for their activity, structural integrity and stability. They belong to family 13 (GH-13) of the glycoside hydrolase group of enzymes. The α-amylases family can be divided in to two groups that are the starch hydrolyzing enzymes and the starch modifying or transglycosylating enzymes (De Souza and Magalhaes, 2010).

Another form of amylase, β-amylase (EC 3.2.1.2; 1,4-β-D-glucan maltohydrolase) is also synthesized by bacteria, fungi and plants. Working from the non-reducing end, β-amylase catalyzes the hydrolysis of the second β-1,4 glycosidic bond, cleaving off two glucose units (maltose) at a time. During the ripening of fruit, β-amylase breaks starch into sugar, resulting in the sweet flavor of ripe fruit. Both are present in seeds, β-amylase is present prior to germination whereas β-amylase and proteases appear once germination has begun. Cereal grain amylase is key to the production of malt. Many microbes also produce amylase to degrade extracellular starches. Animal tissues do not contain β-amylase, although it may be present in microorganisms contained within the digestive tract.

In addition to cleaving the last γ (1-4) glycosidic linkages at the nonreducing end of amylose and amylopectin, yielding glucose. γ-amylase (EC 3.2.1.3, Glucan 1,4-γ-glucosidase) will cleave γ (1-6) glycosidic linkages (Sivaramakrishnan *et al*.,

2006). Unlike the other forms of amylase, γ-amylase is most efficient in acidic environments and has an optimum pH of 3 (Simon *et al*., 1991).

2.6 Application of amylases

The history of the industrial production of enzymes dates back to the time when Dr. Jhokichi Takamine began the production of digestive enzyme preparation by wheat bran koji culture of *Aspergillus oryzae* in 1894. Industrial production of dextrose powder and dextrose crystals from starch using α-amylase and glucoamylase began in 1959. Since then, amylases are being used for various purposes. Conversion of starch into sugar, syrups and dextrins forms the major part of the starch processing industry (Marshall, 1975). The hydrolysates are used as carbon sources in fermentation as well as sources of sweetness in a range of manufactured food products and beverages. Hydrolysis of starch to products containing glucose, maltose, etc. is brought about by controlled degradation (Abu *et al*., 2005).

Thermostable and thermotolerant amylases have a great significance with extensive biotechnological applications in bread and baking, food, textile, and paper industries (Pandey *et al*., 2000). Amylases having approximately 30% of the enzyme market (Burhan, 2003) have almost completely replaced chemical hydrolysis of starch in starch processing industry (Pandey *et al*., 2000). Thermostable and thermotolerant amylases have had extensive commercial applications in starch processing, brewing and sugar production (Leveque, 2000),

desizing in textile industries (Hendriksen, 1999) and in detergent manufacturing processes (Hewitt, 1996).

2.6.1 Bread making

Bread is the most common and traditional foods around the world. But bread actually has close links with enzymes. For years, enzymes such as malt and fungal alpha-amylase have been used in bread making. Due to the changes in the baking industry and the ever-increasing demand for more natural products, enzymes have gained real importance in bread-making (Granum, 1979). Amylase enzymes are used extensively in bread making to break down complex sugars such as starch (found in flour) into simple sugars. Yeast then feeds on these simple sugars and converts it into the waste products of alcohol and CO_2. This imparts flavour and causes the bread to rise. While amylase enzymes are found naturally in yeast cells, it takes time for the yeast to produce enough of these enzymes to break down significant quantities of starch in the bread. This is the reason for long fermented doughs such as sour dough. Modern bread making techniques have included amyalse enzymes into bread improver thereby making the bread making process faster and more practical for commercial use. Amylases degrade starch and produce small dextrins for the yeast to act. Gluten which is presence in flour is a combination of proteins, which form a large network during dough formation (Arendt *et al*., 2002; Ward and Andon, 2002; Gallagher *et al*., 2004).

2.6.2 Hydrolysis of starch to maltodextrins

Starchy substances constitute the major part of the human diet for most of the people in the world, as well as many other animals. They are synthesized naturally in a variety of plants. Some plant examples with high starch content are corn, potato, rice, sorghum, wheat, and cassava. It is no surprise that all of these are part of what we consume to derive carbohydrates. Starch molecules are glucose polymers linked together by the α-1,4 and α-1,6 glucosidic bonds. In order to make use of the carbon and energy stored in starch, the human digestive system, with the help of the enzyme amylases, must first break down the polymer to smaller assimilasable sugars, which is eventually converted to the individual basic glucose units.

Starch is generally insoluble in water at room temperature. Starch in nature is stored in cells as small granules which can be seen under a microscope. Starch granules are quite resistant to penetration by both water and hydrolytic enzymes due to the formation of hydrogen bonds within the same molecule and with other neighboring molecules. These inter- and intra-hydrogen bonds can become weak as the temperature of the suspension is raised. Thus thermostable and thermotolerant amylases are the best hydrolytic enzymes for starch hydrolysis because of their thermostability characteristic (Konsula and Liakopoulou-Kyriakides, 2004).

2.6.3 Sugar industry

Sugar is a class of edible crystalline substances, mainly sucrose, lactose, and fructose. Human taste buds interpret its flavor as sweet. Sugar as a basic food carbohydrate primarily comes from sugar cane and sugar beet, but also appears in fruit, honey, sorghum, sugar maple, and in many other sources. Table sugar (sucrose) comes from plant sources. Two important sugar crops predominate are sugarcane and sugar beets, in which sugar can account for 12% to 20% of the plant's dry weight. Some minor commercial sugar crops include the date palm, sorghum, and the sugar maple. The sugar industry processes sugar cane and sugar beet to manufacture edible sugar with more than 60% of the world’s sugar production is from sugar cane and the balance is from sugar beet.

In sugar processing, the first step includes gelatinization of the starch slurry which is achieved by heating starch with water at temperatures around 100 ℃, due to insolubility of starch at lower temperatures. This step involves dissolution of starch granules thereby forming a viscous suspension. Because of this high viscosity it poses serious problems with mixing and pumping (Soni *et al*., 2005; Kunamneni and Singh, 2005b). To overcome these viscosity-associated problems, gelatinization is coupled with liquefaction which involves partial hydrolysis and loss in viscosity. This action is brought about by thermostable α-amylases (Crabb and Mitchinson, 1997), which can act at temperatures around 70-100 ℃ depending on the temperature profile of the α-amylase (Soni *et al*., 2003). Further steps in

sugar processing include saccharification, involving the production of glucose and maltose via further hydrolysis. As a result, thermostable α-amylases are gaining wide industrial and biotechnological interest because their enzymes are better suited for harsh industrial processes and in addition, their application reduces contamination risk and reaction time (Schoonees, 2004; Eggleston *et al*., 2008).

2.6.4 Paper industry

The pulp and paper industry is a mature sector of the chemical industry that remains critically important for many countries and regions of the world including the U.S. The paper industry has been searching for new and innovative solutions to reduce its operating costs and make paper production operations more profitable. Such process improvement solutions will have a better chance of being adopted by the industry if they can provide additional environmental benefits and allow the industry to comply with stringent environmental regulations and meet the public demand for protection of natural resources.

The use of amylases in the pulp and paper industry is in the modification of starch for coated paper especially for the production of low-viscosity, high molecular weight starch (Gupta *et al*., 2003). The coating treatment serves to make the paper surface become sufficiently smooth and strong,besides to improve the writing quality of the paper (Bruinenberg *et al*., 1996). Starch is also a good sizing agent coating paper for the finishing paper, improving the quality and erasebility, besides being a good coating for the paper. The sizing of paper is performed to

protect the paper against mechanical damage during processing and also improves the quality of the finished paper. The viscosity of natural starch is too high for paper sizing and this can be altered by partially degrading the polymer with α-amylases (De Souza and Magalhaes, 2010). A number of amylases obtained from fungi are used in paper industry and these include thermostable α-amylase G995 (Enzyme Biosystem, USA).

2.6.5 Textile desizing

In textile weaving, especially for fabric made of cotton or blends the warp threads are coated with an adhesive substance known as 'size'; to prevent the threads from breaking during weaving. Although many different compounds have been used to size fabrics, starch and its derivatives have been the most common sizing agent and it could be due to the cheap, easily available and itcan be removed quite easily. Starch paste is applied for warping and this will give strength to the textile at the weaving process. Besides, it also prevents the loss of string due to friction, cutting and generation of static electricity on the string by giving softness to the surface of string due to laid down warp. After weaving, the starch (size) is removed from the cloth and the cloth is then goes to scouring and dyeing and finally finishing stages. The α-amylase is usually used to remove the starch from the cloth (Aiyer, 2004) without attacking the fibers (Feikenhauer, 2003; Gupta *et al*., 2003; Ahlawat *et al.,* 2009; De Souza and Magalhaes, 2010).

This process (desizing) must be carried out by treating the fabric with chemicals

such as acids, alkali or oxidizing agents. However starch-breaking enzymes (amylases) are preferred for desizing due to their high efficiency and specific action. Amylases bring about complete removal of the size without any harmful effects on the fabric. Another benefit of enzymes compared to strong chemicals mentioned above is that enzymes are environment friendly.

2.6.6 Detergent industry

Detergent industry is the primary consumers of enzymes, in term of value and volume. The use of enzymes in detergent formulations enhances the detergent ability to remove tough stains and making the detergent environmentally safe. Amylases are used to remove residues of starch-based foods like potatoes, spaghetti, custards, gravies, chocolate and so on to dextrins and other smaller oligosaccharides (Olsen and Falholt, 1998; Mukherjee *et al.,* 2009). Amylases are the second type of enzymes used in the formulation of enzymatic detergent and about 90% of all liquid detergent contain these enzymes (Gupta *et al.,* 2003; Hmidet *et al*., 2009; Mitidieri *et al.,* 2006). This enzyme is used in laundry detergents as well as in dishwashing detergents (Jung *et al*., 2003). Amylases have activity at lower temperature and alkaline pH, maintaining the necessary stability under detergent conditions and the oxidative stability of amylases is one of the most important criteria for their use in detergents where the washing environment is very oxiding (Kirk *et al*., 2002).